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(54) Title: $\alpha(1,3)$ GALACTOSYLTRANSFERASE NEGATIVE SWINE			
(57) Abstract			
<p>Transgenic swine in which the normal expression of <math>\alpha(1,3)</math> galactosyltransferase is prevented in at least one organ of tissue type. The absence or inactivation of this enzyme prevents the production of carbohydrate moieties having the distinctive terminal Gal<math>\alpha</math>1-3Gal<math>\beta</math>1-4GlcNAc epitope that is a significant factor in xenogeneic, particularly human, transplant rejection of swine grafts.</p> <p style="text-align: right;"> <i>injected</i>  <i>no</i>  <i>actual</i>  <i>analysis</i>  <i>monoclonal</i>  <i>no example of the cells</i> </p>			

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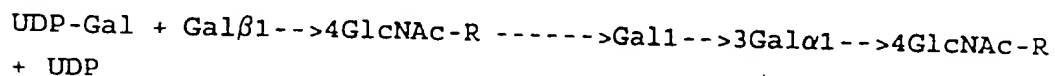
$\alpha$ (1,3) GALACTOSYLTRANSFERASE NEGATIVE SWINE

Donor organ shortages have led to hopes that xenotransplantation could serve as an alternative means of organ availability. Swine, particularly mini-swine, are an attractive alternative to non-human primate donors because of potentially greater availability, the reduced risk of zoonotic infections, appropriate size of organs and the reduced social and ethical concerns (Sachs, D.H. et al. 1976. Transplantation 22:559-567; Auchincloss, H. Jr. 1988. Transplantation 46:1-20). However, one of the major barriers to xenotransplantation is the phenomenon described as hyperacute rejection (Busch et al. 1972. Am. J. Pathology 79:31-57; Auchincloss, H. Jr. 1988. Transplantation 46:1-20). This phenomenon describes a very rapid and severe humoral rejection, which leads to destruction of the graft within minutes or hours of the transplant of the donor organ. Hyperacute rejection is apparently mediated by a complex series of events, including activation of the complement systems, activation of blood coagulation proteins, activation of endothelial cells and release of inflammatory proteins (Busch et al. 1972. Am. J. Pathology 79:31-57; Platt, J. L. 1992. ASAIO Journal 38:8-16). There is an accumulating body of information that implicates a group of pre-formed antibodies, the so-called natural antibodies, to be of

fundamental importance in the hyperacute rejection seen in grafts between species. Species combinations in which the recipients of grafts have circulating antibodies that can initiate the hyperacute response to the donor species are described as discordant. Pigs and humans are one such discordant species combination.

The hyperacute rejection process is initiated when the natural antibodies of the recipient bind to cells of the donor organ (Platt et al. 1990. Transplantation 50:870-822; Platt et al. 1990. Immunology Today 11:450-456). It has been suggested that porcine N-linked carbohydrates carrying a terminal Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc structure are the major targets for anti-swine xenoreactive human natural antibodies (Good et al. 1992. Transplantation Proceedings 24:559-562; Sandrin et al. 1993. Proc. Natl. Acad. Sci. USA 90:11391-11395). One major difference between the glycosylation pattern of swine tissues and human tissues is the presence of high levels of a terminal Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc structure on swine cells and tissues. This structure is expressed at high levels in all lower mammals investigated, but is poorly expressed on cells and tissues of Old World monkeys, apes and humans (catarrhines) (Galili, U. and Swanson, K. 1992. Proc. Natl. Acad. Sci. USA 88:7401-7404; Galili et al. 1987. Proc. Natl. Acad. Sci. USA. 84:1369-1373). A specific transferase, UDP-Gal:Gal $\beta$ 1-->4GlcNAc  $\alpha$ 1-->3-galactosyltransferase (EC 2.4.1.151;  $\alpha$ (1,3) galactosyltransferase) is responsible for the transfer of a terminal galactose to the terminal galactose residue of N-acetylactosamine-type carbohydrate chains and lactosaminoglycans according to the reaction:

$Mn^{2+}$



where R may be a glycoprotein or a glycolipid (Blanken, W. M. and Van den Eijnden, D. H. 1985. J. Biol. Chem. 260:12927-12934). Thus the Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc epitope. Full length cDNA sequences encoding the murine (Larsen et al. 1989. Proc. Natl. Acad. Sci. USA. 86:8227-8231) and bovine (Joziassse et al. 1989. J. Biol. Chem. 264:14290-14297) enzymes have been determined. In addition, the genomic organization of the murine  $\alpha$ (1,3) galactosyltransferase gene has been established (Joziassse et al. 1992. J. Biol. Chem. 267:5534-5541). A partial sequence encoding the 3' region of the porcine  $\alpha$ (1,3) galactosyltransferase cDNA gene has been determined (Dabkowski et al. 1993. Transplantation Proceedings. 25:2921) but the full length sequence has not been reported. The absence of the 5' sequence is significant for the applications described herein. In contrast to the lower mammals, humans do not express the  $\alpha$ (1,3) galactosyltransferase. Furthermore, human sequences homologous to the murine sequence correspond to a processed pseudogene on chromosome 12 and an inactivated remnant on chromosome 9 (Shaper et al. 1992. Genomics 12:613-615).

In accordance with the invention, swine organs or tissues or cells that do not express  $\alpha$ (1, 3) galactosyltransferase will not produce carbohydrate moieties containing the distinctive terminal Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc epitope that is a significant factor in xenogeneic, particularly human, transplant rejection of swine grafts. Further in accordance with the invention, is the aspect of diminishing the production of  $\alpha$ (1,3) galactosyltransferase to an extent sufficient to prevent the amount produced from providing carbohydrates with the Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc epitope from being presented to the cell surface thereby rendering the transgenic animal, organ, tissue, cell or cell culture immunogenically tolerable to the intended recipient without

requiring complete  $\alpha(1,3)$  galactosyltransferase gene suppression.

One principal aspect of the present invention is that the inventors have isolated the entire porcine  $\alpha(1,3)$  galactosyltransferase cDNA gene (SEQ. ID NO. 1). The identification, isolation and sequencing of the entire cDNA gene, now particularly providing the sequence of the 5' end is an important advance because, as described in Example 2, this region has been identified as the most efficient for antisense targeting. Moreover, as compared with mouse and bovine homologous sequences (Figure 2), this region of the  $\alpha(1,3)$  galactosyltransferase mRNA appears to deviate extensively between these species making it extremely unlikely that a use of "cross-species" antisense constructs would be successful.

Another principle aspect of this invention related to genetically altered animals, more specifically transgenic, chimeric or mosaic swine in which the expression of biologically active  $\alpha(1, 3)$  galactosyltransferase is prevented in at least one organ, tissue or cell type. Transgenic animals carry a gene which has been introduced into the germline of the animal, or an ancestor of the animal, at an early developmental stage. The genetic alteration in transgenic animals is stably incorporated into the genome as a result of intentional experimental intervention. Typically, this results from the addition of exogenous foreign DNA or novel constructs (Palmiter et al. 1986. Ann. Rev. Genet. 20:465). With the advent of embryonic stem (ES) cells and specific gene targeting, the definition of transgenesis now includes specific modification of endogenous gene sequences by direct experimental manipulation and by stable incorporation of DNA that codes for effector molecules that modulate the expression of endogenous genes

(Gossler et al. 1986. Proc. Natl. Acad. Sci. USA. 83:9065; Schwarzberg et al. 1989. Science 246:799; Joyner et al. 1989. Nature 338:153).

One preferred approach for generating a transgenic animal involves micro-injection of naked DNA into a cell, preferentially into a pronucleus of an animal at an early embryonic stage (usually the zygote/one-cell stage). DNA injected as described integrates into the native genetic material of the embryo, and will faithfully be replicated together with the chromosomal DNA of the host organism. This allows the transgene to be passed to all cells of the developing organism including the germ line. Transgene DNA that is transmitted to the germ line gives rise to transgenic offspring. If transmitted in a Mendelian fashion, half of the offspring will be transgenic. All transgenic animals derived from one founder animal are referred to as a transgenic line. If the injected transgene DNA integrates into chromosomal DNA at a stage later than the one cell embryo not all cells of the organism will be transgenic, and the animal is referred to as being genetically mosaic. Genetically mosaic animals can be either germ line transmitters or non-transmitters. The general approach of microinjection of heterologous DNA constructs into early embryonic cells is usually restricted to the generation of dominant effects, i.e., one allele of the transgene (hemizygous) causes expression of a phenotype (Palmiter et al. 1986. Ann. Rev. Genetics 20:465.)

In another preferred approach, animals are genetically altered by embryonic stem (ES) cell-mediated transgenesis (Gossler et al. 1986, Proc. Natl. Acad. Sci. USA. 83:9065). ES cell lines are derived from early embryos, either from the inner cell mass (ICM) of a blastocyst (an embryo at a relatively early stage of development) or migrating

primordial germ cells (PGC) in the embryonic gonads. They have the potential to be cultured in vitro over many passages (i.e. are conditionally immortalized), and they are pluripotent, or totipotent (i.e. are capable of differentiating and giving rise to all cell types. ES cells can be introduced into a recipient blastocyst which is transferred to the uterus of a foster mother for development to term. A recipient blastocyst injected with ES cells can develop into a chimeric animal, due to the contributions from the host embryo and the embryonic stem cells. ES cells can be transfected with heterologous gene constructions that may cause either dominant effects, inactivate whole genes or introduce subtle changes including point mutations. Subsequent to clonal selection for defined genetic changes, a small number of ES cells can be reintroduced into recipient embryos (blastocysts or morulae) where they potentially differentiate into all tissues of the animal including the germ line and thus, give rise to stable lines of animals with designed genetic modifications. Totipotent porcine embryonic stem cells can be genetically altered to have a heterozygous (+/-) mutant, preferably null mutant allele, particularly one produced by homologous recombination in such embryonic stem cells. Alternatively, gene targeting events by homologous recombination can be carried out at the same locus in two consecutive rounds yielding clones of cells that result in a homozygous (-/-) mutant, preferably a null mutant (Ramirez-Solis et al. 1993. Methods in Enzymol. 225:855).

In one preferred embodiment of this invention a DNA sequence is integrated into the native genetic material of the swine and produces antisense RNA that binds to and prevents the translation of the native mRNA encoding  $\alpha(1,3)$  galactosyltransferase in the transgenic swine.



In a particularly preferred embodiment the genome of the transgenic swine is modified to include a construct comprising a DNA complementary to that portion of the  $\alpha(1,3)$  galactosyltransferase coding region that will prevent expression of all or part of the biologically active enzyme. As the term is used "integrated antisense sequence" means a non-native nucleic acid sequence integrated into the genetic material of a cell that is transcribed (constitutively or inducibly) to produce an mRNA that is complementary to and capable of binding with an mRNA produced by the genetic material of the cell so as to regulate or inhibit the expression thereof.

In another embodiment of the invention, cells or cell lines from non-mutant swine are made with the  $\alpha(1,3)$  galactosyltransferase inactivated on one or both alleles through the use of an integrated antisense sequence which binds to and prevents the translation of the native mRNA encoding the  $\alpha(1,3)$  galactosyltransferase in said cells or cell lines. The integrated antisense sequence, such as the RNA sequence transcribed in Example 3 is delivered to the cells by various means such as electroporation, retroviral transduction or lipofection.

In another preferred embodiment, the transgenic swine is made to produce a ribozyme (catalytic RNA) that cleaves the  $\alpha(1,3)$  galactosyltransferase mRNA with specificity. Ribozymes are specific domains of RNA which have enzymatic activity, either acting as an enzyme on other RNA molecules or acting intramolecularly in reactions such as self-splicing or self-cleaving (Long, D.M. and Uhlenbeck, O.C. 1993. FASEB Journal. 7:25-30). Certain ribozymes contain a small structural domain generally of only about 30 nucleotides called a "hammerhead". The hammerhead is a loop of RNA that is flanked by two linear domains that are specific

complements to domains on the substrate to be cleaved. The site on the hammerhead ribozyme that effects the cleavage of substrate is the base of the stem loop or hammerhead. As shown in Figure 3, the ribozymes of the present invention have flanking sequences complementary to domains near the 5' end of the  $\alpha(1,3)$  galactosyltransferase cDNA gene.

The DNA for the ribozymes is integrated into the genetic material of an animal, tissue or cell and is transcribed (constitutively or inducibly) to produce a ribozyme which is capable of selectively binding with and cleaving the  $\alpha(1,3)$  galactosyltransferase mRNA. As it is a catalytic molecule, each such ribozyme is capable of cleaving multiple substrate molecules.

The catalytic "stem loop" of the ribozyme is flanked by sequences complementary to regions of the  $\alpha(1,3)$  galactosyltransferase mRNA. In a particularly preferred embodiment the transgenic swine is modified to integrate a construct comprising the DNA coding for that portion of catalytic RNA necessary to inactivate the mRNA of the  $\alpha(1,3)$  galactosyltransferase operably linked to a promoter therefor.

In another embodiment of the invention, cells or cell lines from non-mutant swine are made with the  $\alpha(1,3)$  galactosyltransferase inactivated on one or both alleles through the use of an integrated ribozyme sequence which binds to and cleaves the native mRNA encoding the  $\alpha(1,3)$  galactosyltransferase in said cells or cell lines. The integrated ribozyme sequence, such as the RNA sequence transcribed in Example 4 is delivered to the cells by various means such as electroporation, retroviral transduction or lipofection.

In another preferred embodiment, using cultured porcine embryonic stem cells, a mutation, preferably a null mutation is introduced by gene targeting at the native genomic locus encoding  $\alpha(1,3)$  galactosyltransferase. Gene targeting by homologous recombination in ES cells is performed using constructs containing extensive sequence homology to the native gene, but specific mutations at positions in the gene which are critical for generating a biologically active protein. Therefore, mutations can be located in regions important for either translation, transcription or those coding for functional domains of the protein. Selection for ES clones that have homologously recombined a gene targeting construct, also termed gene "knock out" construct, can be achieved using specific marker genes. The standard procedure is to use a combination of two drug selectable markers including one for positive selection (survival in the presence of drug, if marker is expressed) and one for negative selection (killing in the presence of the drug, if marker is expressed) (Mansour et al., 1988. Nature 336:348). One preferred type of targeting vector includes the neomycin phosphotransferase (neo) gene for positive selection in the drug G418, as well as the Herpes Simplex Virus-thymidine kinase (HSV-tk) gene for selective killing in gancyclovir. Drug selection in G418 and gancyclovir, also termed positive negative selection (PNS) (Mansour et al. 1988. Nature 336:348; Tubulewicz et al. 1991. Cell 65:1153) allows for enrichment of ES cell clones that have undergone gene targeting, rather than random integration events. Confirmation of homologous recombination events is performed using Southern analysis.

The design of the  $\alpha(1,3)$  galactosyltransferase targeting construct is described in Example 6. The procedure as applied here uses a positive selection (survival) based on integration of the neo (neomycin resistance), preferably in

inverse orientation to the endogenous  $\alpha(1,3)$  galactosyltransferase gene locus in a cassette with the phosphoglycerate kinase (PGK-1) promoter and with flanking oligonucleotides complementary to two separate regions of the  $\alpha(1,3)$  galactosyltransferase gene sequence. It is understood that other positive selectable markers may be used instead of neo. The neo gene is linked with its promoter to be under control thereof. Downstream from the second flanking sequence is the HSV-tk gene which, if integrated into the genome encodes for production of thymidine kinase making the cell susceptible to killing by gancyclovir (negative selection). The integration of the neo gene but not the HSV-tk gene occurs only where integration into the  $\alpha(1,3)$  galactosyltransferase gene has occurred and provides for both positive and negative selection of the cells so transformed.

In another preferred embodiment, using isogenic DNA, it has become possible to achieve high frequency homologous recombination even in biological systems, such as zygotes, which do not lend themselves to the use of elaborate selection protocols and were, therefore, previously not suitable candidates for the isolation of cells which showed positive marker attributes indicating homologous recombination. This use of targeting vectors which include isogenic DNA which is substantially identical to that of chromosomal segments of the target recipient cell, can be used to target zygotes which thereafter develop into transgenic animals. The use of these zygote cells is preferably to produce a mutation, preferably a null mutation, at the chromosomal locus encoding  $\alpha(1,3)$  galactosyltransferase. Thus, these vectors contain extensive sequence homology to the native gene, but also contain specific mutations at segments in the gene which are critical for generating a biologically active protein. Therefore, mutations can be located in regions important for either

translation, transcription, or those coating for functional domains of the protein. The high percentage of homologous recombination achieved using isogenic DNA makes it possible to avoid the need for selection of clones that have homologously recombined the gene targeting construct, as described above with respect to the "knock out" embodiment thereby making it possible to avoid the need for the standard selection procedure described above.

More particularly, in this embodiment, PCR is used to identify and extract 1-2 kb DNA fragments, which are then subjected to restriction fragment length polymorphism digestions to identify areas or alleles that are most abundantly present in the line of mini-swine selected for zygote injection. Known insertion vectors are used, however, replacement vectors also described in the art could also be used. It is also possible to use DNA sequences with an isogenic replacement vector that require only a few kilobases of uninterrupted isogenic DNA. As such, it is possible to effect highly efficient homologous recombination such that it is not necessary to screen targeting vector recombined zygotes. Rather, genomic DNA screening using PCR can be done after the piglets are born. These transgenic founder animals, which are observed to have the transgene targeted to the native  $\alpha$  (1,3) galactosyltransferase locus (i.e. that are heterozygous null mutant for that gene, are grown and interbred to produce animals that are homozygous null mutant for this locus, resulting in the knock out of the  $\alpha$  (1,3) galactosyltransferase gene which can be confirmed by antibody or lectin binding assays.

The swine is preferably an  $\alpha$ (1,3) galactosyltransferase negative swine grown from a porcine oocyte whose pronuclear material has been removed and into which has been introduced a totipotent porcine embryonic stem cell using protocols for

nuclear transfer (Prather et al. 1989, Biol. Reprod. 41:414) ES cells used for nuclear transfer are negative for the expression of  $\alpha(1,3)$  galactosyl transferase, or alternatively, totipotent ES cells used for nuclear transfer are mutated in a targeted fashion in at least one allele of the  $\alpha(1,3)$  galactosyltransferase gene.

The swine is preferably lacking expression of the  $\alpha(1,3)$  galactosyltransferase gene and bred from chimeric animals which were generated from ES cells by blastocyst injection or morula aggregation. ES cells used to generate the preferably null-mutated chimeric animal were mutated at least in one allele of the  $\alpha(1,3)$  galactosyltransferase gene locus, using gene targeting by homologous recombination.

A chimeric swine is preferably constituted by ES cells mutated in one allele of the  $\alpha(1,3)$  galactosyltransferase gene. Derived from mutated ES cells are also germ cells, male or female gametes that allow the mutation to be passed to offspring, and allow for breeding of heterozygous mutant sibling pigs to yield animals homozygous mutant at the  $\alpha(1,3)$  galactosyltransferase locus. Also described is a swine, deficient for an  $\alpha(1,3)$  galactosyltransferase protein (i.e., characterized by lack of expression of  $\alpha(1,3)$  galactosyltransferase protein) and have little, if any, functional Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc epitope-containing carbohydrate antigen on the cell surface are produced. Further described are methods of producing transgenic swine and methods of producing tissue from heterozygous swine or homozygous swine of the present invention. The present invention also relates to cell lines, such as swine cell lines, in which the  $\alpha(1,3)$  galactosyltransferase gene is inactivated on one or both alleles and use of such cell lines as a source of tissue and cells for transplantation.

Tissues, organs and purified or substantially pure cells obtained from transgenic swine, more specifically from hemizygous, heterozygous or homozygous mutant animals of the present invention can be used for xenogeneic transplantation into other mammals including humans in which tissues, organs or cells are needed. The  $\alpha(1,3)$  galactosyltransferase inactive cells can themselves be the treatment or therapeutic/clinical product. For example, keratinocytes rendered  $\alpha(1,3)$  galactosyltransferase inactive can be used for macular degeneration and pancreatic cells rendered  $\alpha(1,3)$  galactosyltransferase deficient can be used to replace or restore pancreatic products and functions to a recipient. In another embodiment,  $\alpha(1,3)$  galactosyltransferase inactive cells produced by the present method are further manipulated, using known methods, to introduce a gene or genes of interest, which encode(s) a product(s), such as a therapeutic product, to be provided to a recipient. In this embodiment, the  $\alpha(1,3)$  galactosyltransferase deficient tissue, organ or cells serve as a delivery vehicle for the encoded product(s). For example,  $\alpha(1,3)$  galactosyltransferase deficient cells, such as fibroblasts or endothelial cells, can be transfected with a gene encoding a therapeutic product, such as cytokines that augment donor tissue engraftment, Factor VIII, Factor IX, erythropoietin, insulin, human major histocompatibility (MHC) molecules or growth hormone, and introduced into an individual in need of the encoded product.

Alternatively, recipient blastocysts are injected or morulae are aggregated with totipotent embryonic stem cells yielding chimeric swine containing at least one allele of a mutated, preferably null-mutated  $\alpha(1,3)$  galactosyltransferase gene produced by homologous recombination. A chimeric swine is preferably constituted by ES cells mutated in one allele of the  $\alpha(1,3)$  galactosyltransferase gene. Derived from mutated ES cells are also germ cells that allow the mutation

to be passed to offspring, and breeding of heterozygous mutant sibling pigs to yield animals homozygous mutant at the  $\alpha(1,3)$  galactosyltransferase locus. Also described is a swine, deficient for an  $\alpha(1,3)$  galactosyltransferase protein (i.e., characterized by essentially no expression of  $\alpha(1,3)$  galactosyltransferase protein) and with little, if any, functional Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc epitope-containing carbohydrate antigen on the cell surface are produced. Further described are methods of producing transgenic swine and methods of producing tissue from heterozygous swine or homozygous swine of the present invention. The present invention also related to cell lines, such as swine cell lines, in which the  $\alpha(1,3)$  galactosyltransferase gene is inactivated on one or both alleles and use of such cell lines as a source of tissue, organs and cells for transplantation.

Figure 1 illustrates the complete cDNA sequence of the  $\alpha(1,3)$  galactosyltransferase gene (SEQ. ID. No. 1), having an open reading frame of 1113 base pairs, encoding a 371 amino acid protein.

Figure 2 compares the protein sequences encoded by the porcine, bovine and murine  $\alpha(1,3)$  galactosyltransferase cDNA genes.

Figure 3 illustrates the secondary structure of a trans-acting hammerhead ribozyme targeted to  $\alpha(1,3)$  galactosyltransferase mRNA.

Figure 4 illustrates the genomic organization of the murine  $\alpha(1,3)$  galactosyltransferase gene. Exons are labeled 1-9 and are indicated by solid boxes. Intron sequences are represented by a thin line.



A method of producing a chimeric swine and porcine organs and tissue cultures, homozygous for an  $\alpha(1,3)$  galactosyltransferase gene inactivation, in which  $\alpha(1,3)$  galactosyltransferase protein synthesis and cell surface Gal $\alpha(1-3)$ Gal $\beta(1-4)$ GlcNAc epitope-containing carbohydrate cell surface markers expression are deficient is disclosed. Of particular interest are purified cell types which have been rendered ~~NOT DEFICIENT~~ ~~IN~~ ~~THE~~  $\alpha(1,3)$  galactosyltransferase expression. Such cell types include fibroblasts, keratinocytes, myoblasts and endothelial cells.

In one embodiment of the present invention, swine cells altered as described herein are used to provide cells needed by a recipient or to provide gene therapy. The cells, which are deficient in Gal $\alpha(1-3)$ Gal $\beta(1-4)$ GlcNAc epitope-containing carbohydrates cell surface antigen, are cultured and transplanted to an oocyte.

The embryonic stem cells with the null mutant  $\alpha(1,3)$  galactosyltransferase locus are introduced into swine blastocysts, which are then introduced into a pseudopregnant swine. The embryo develops into a chimeric swine offspring. When bred with wild-type females, chimeric males transmit the  $\alpha(1,3)$  galactosyltransferase inactivation in the embryonic stem cell to their offspring, which are heterozygous for the inactivation. Swine heterozygous for the  $\alpha(1,3)$  galactosyltransferase gene inactivation can be intercrossed to produce swine homozygous (-/-) for the mutation.

Purified or substantially pure  $\alpha(1,3)$  galactosyltransferase deficient cells can be obtained from tissues or transgenic or chimeric swine produced as described herein. Alternatively, they can be obtained from a normal (non-altered) donor swine and altered using a method described herein. These cells can be then cultured by known

methods to produce a quantity of cells useful for transplantation. In addition, cell lines, such as swine cell lines, in which the  $\alpha(1,3)$  galactosyltransferase gene is disrupted, preferably on both alleles, are useful as a source of tissue and cells for transplantation.

#### EXAMPLE 1

##### ISOLATION AND CHARACTERIZATION OF PORCINE $\alpha(1,3)$ GALACTOSYLTRANSFERASE cDNA

A previously described  $\lambda$ ZAP II porcine spleen cDNA library (Gustafsson et al. 1990. Proc. Natl. Acad. Sci. USA. 87:9798-9802) was screened by hybridization with a cloned  $\alpha(1,3)$  galactosyltransferase cDNA probe (Joziassse et al. 1989. J. Biol. Chem. 264:14290-14297). The Genbank Accession number for the bovine  $\alpha(1,3)$  galactosyltransferase cDNA sequence is J04989. The bovine  $\alpha(1,3)$  galactosyltransferase cDNA probe was kindly provided by Dr. David Joziassse, University of Leiden, The Netherlands. The probe was radioactively labeled with a  $^{32}\text{P}$ -dATP using the Megaprime DNA labeling system (Amersham International, UK). Positive clones were confirmed by the polymerase chain reaction (PCR), using primers (SEQ. ID. NO: 2 and SEQ. ID. NO: 3) derived from the bovine  $\alpha(1,3)$  galactosyltransferase cDNA sequence. SEQ. ID. NO: 2 corresponds to bovine  $\alpha(1,3)$  galactosyltransferase nucleotides 712-729 and SEQ. ID. NO: 3 corresponds to the reverse complement of bovine  $\alpha(1,3)$  galactosyltransferase nucleotides 1501-1508. Recombinant pBluescript plasmids from positive clones were automatically excised with the helper phage R408 (Stratagene Ltd., Cambridge, UK) and amplified in *E. coli* strain TG1 (ATCC 39078). Plasmid DNA was prepared using the Magic Miniprep kit (Promega Ltd., Southampton, UK) following the manufacturer's instructions and the DNA was characterized by cleavage with EcoRI. DNA sequencing was performed by the

dideoxy chain termination method, using a T7 DNA polymerase sequencing kit (Pharmacia Biosystems Ltd., Milton Keynes, UK) according to the manufacturer's instructions. The synthetic oligonucleotide primers SEQ. ID. NOs. 4-12 were used. SEQ. ID. NO. 4 is Stratagene SK, catalog number 300305, (Stratagene Inc., La Jolla, CA). SEQ. ID. NO: 5 corresponds to the reverse complement of porcine  $\alpha(1,3)$  galactosyltransferase nucleotides 94-111. SEQ. ID. NO: 6 corresponds to the porcine  $\alpha(1,3)$  galactosyltransferase nucleotides 163-180. SEQ. ID. NO: 7 corresponds to the reverse complement of porcine  $\alpha(1,3)$  galactosyltransferase nucleotides 442-459. SEQ. ID. NO: 8 corresponds to the complement of porcine and bovine  $\alpha(1,3)$  galactosyltransferase nucleotides 538-555 and 982-999, respectively. SEQ. ID. NO: 9 corresponds to the reverse complement of porcine  $\alpha(1,3)$  galactosyltransferase nucleotides 596-615. SEQ. ID. NO: 10 corresponds to the porcine  $\alpha(1,3)$  galactosyltransferase nucleotides 682-699. SEQ. ID. NO: 11 corresponds to the porcine  $\alpha(1,3)$  galactosyltransferase nucleotides 847-864. SEQ. ID. NO: 12 corresponds to the reverse complement of porcine  $\alpha(1,3)$  galactosyltransferase nucleotides 970-987.

Four positive clones were obtained from approximately  $2 \times 10^4$  plaques screened by hybridization with the bovine  $\alpha(1,3)$  galactosyltransferase cDNA probe (Joziassse et al. 1989. J. Biol. Chem. 264:14290-14297). Three of these clones were confirmed to be positive by PCR. Each of the three recombinant pBluescript plasmids, generated by automatic excision from  $\lambda$ ZapII with helper phage, contained inserts of approximately 2.5 kb as determined by EcoRI cleavage. One clone, designated pS $\alpha$ 13GT1, was selected for further study.

DNA sequence analysis of pS $\alpha$ 13GT1 revealed an open reading frame of 1113 bases (See SEQ. ID. NO: 1 and Figure 1) showing 86% identity with the published bovine cDNA sequence

(Joziassse et al. 1989. J. Biol. Chem. 264:14290-14297) and encoding a 371 amino acid protein with 85% and 76% identity with the bovine and murine  $\alpha(1,3)$  galactosyltransferase amino acid sequences, respectively (Figure 2).

## EXAMPLE 2

### ANTISENSE OLIGONUCLEOTIDE INHIBITION OF $\alpha(1,3)$ GALACTOSYLTRANSFERASE EXPRESSION

Three antisense 5' and 3' phosphothioate-protected oligonucleotides (S-oligonucleotides, SEQ. ID. NOS 13-15) are tested in an *in vitro* system employing porcine primary endothelial cell cultures (from porcine aorta) and a porcine B-cell line (L231, European Collection of Animal Cell Cultures, PHLS, Center for Applied Microbiology and Research, Porton Down, Salisbury, UK). SEQ. ID. NO: 13 corresponds to the reverse complement of porcine  $\alpha(1,3)$  galactosyltransferase cDNA nucleotides 16-35. SEQ. ID. NO: 14 corresponds to the reverse complement of porcine  $\alpha(1,3)$  galactosyltransferase cDNA nucleotides 31-53. SEQ. ID. NO: 15 corresponds to the reverse complement of porcine  $\alpha(1,3)$  galactosyltransferase cDNA nucleotides 6-23. All three antisense oligonucleotides are directed at the 5' region of the mRNA surrounding the initiation of translation. Nonsense S-oligonucleotides randomized from the antisense sequence are used as controls at similar molar concentrations.

Porcine endothelial cells are derived from miniature swine aorta by scraping the luminal surface of the blood vessel as described (Ryan et al. Tissue and Cell 12:619-635). The cells are suspended in M199 medium supplemented with 20% fetal bovine serum (GIBCO BRL, Gaithersburg, MD) and gentamycin and plated in 25 cm<sup>2</sup> tissue culture flasks, pre-coated with fibronectin (5  $\mu$ g/cm<sup>2</sup>) and laminin (1  $\mu$ g/cm<sup>2</sup>). Endothelial cell growth supplement (Collaborative Research,

Bedford, MA) at 150  $\mu$ g/ml is added at the beginning of the culture. The cultures are maintained by changing one half of the medium every 2-3 days. The porcine lymphoblastoid cell line L231 is maintained in DMEM, 10% fetal bovine serum, 10% NCTC-109, 1% glutamine, 1% pen-strep (all from GIBCO BRL, Gaithersburg, MD) and  $5 \times 10^{-3}$  M 2-mercaptoethanol (Sigma, St. Louis, MO). The L231 cells are sub-cultured by splitting the cells 1:3 every three days.

For cell treatment, S-oligonucleotides are added, to a final concentration of 5-10  $\mu$ M, to growing cells at 24 hr intervals, typically for 48 hr, and then the treated cells are examined for the levels of  $\alpha(1,3)$  galactosyltransferase mRNA (by Northern blot analysis) and expression of the epitope on the cell surface by human AB serum and FITC-labeled mouse anti-human secondary reagents (anti-human IgM and IgG).

### EXAMPLE 3

#### PREPARATION AND USE OF INTEGRATED ANTISENSE CONSTRUCTS

We are studying the ability of integrated antisense constructs to inhibit specifically the production of the porcine  $\alpha(1,3)$  galactosyltransferase. Specific inhibition of the  $\alpha(1,3)$  galactosyltransferase in transfected cells allows assessment of the contribution of the enzyme in the hyperacute phenomenon. Vectors are constructed to express the  $\alpha(1,3)$  galactosyltransferase antisense mRNA, under the control of the cytomegalovirus (CMV) promoter. Specifically, pS $\alpha$ 13GT1 is cleaved with NotI and EcoRV which generates a restriction fragment of length 537 bp, containing part of the pBluescript polylinker sequence through to nucleotide 531 of the  $\alpha(1,3)$  galactosyltransferase cDNA sequence. This DNA fragment is cloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA), cleaved with the same enzymes.

The resulting vector therefore contains the porcine  $\alpha(1,3)$  galactosyltransferase sequence in the antisense direction, relative to the CMV promoter located in pcDNA3. The construct is transfected, using electroporation or other high efficiency methods for introducing DNA into mammalian cells, into both porcine endothelial cells and the L231 porcine lymphoblastoid cell lines (grown as described in Example 2). The effect of the antisense RNA is monitored by both Northern blot analysis of mRNA of the  $\alpha(1,3)$  galactosyltransferase gene and the degree of binding of human serum components (i.e., natural antibodies).

#### EXAMPLE 4

##### RIBOZYME SEQUENCES THAT INACTIVATE PORCINE $\alpha(1,3)$ GALACTOSYLTRANSFERASE mRNA

This example describes a method for construction of the vectors which encode ribozyme sequences which are specifically designed to cleave the porcine  $\alpha(1,3)$  galactosyltransferase mRNA sequence.

The design of the ribozyme sequences is based upon the consensus cis-acting hammerhead ribozyme sequence (Ruffner et al. 1990. Biochemistry 29:10695-10702). We used the Zuker algorithm in the suite of programs available from the Genetics Computer Group (Madison, WI.) to model the cis-acting hammerhead ribozyme sequences (Denman, R.B. 1993. BioTechniques 15:1090-1095). Ribozyme target sequences are identified within the  $\alpha(1,3)$  galactosyltransferase mRNA sequence. A ribozyme sequence file for each potential ribozyme sequence is generated based on the target sequence and using five nucleotides to connect the mRNA target sequence with the catalytic strand of the ribozyme. The sequence file is then folded into the lowest energy structure using RNAFOLD. Sequences which have non-ribozyme structures

are discarded. Figure 3 illustrates one of the ribozyme-target RNA secondary structures, using the ribozyme corresponding to SEQ. ID. NO. 16. The small arrow indicates the cleavage site on the mRNA, between stem I and stem III.

Synthetic oligonucleotides to encode the ribozymes (SEQ. ID. NOS. 16-21) are made on an Applied BioSystems Oligonucleotide synthesizer (Foster City, CA) with termini corresponding to the overhangs of the restriction endonucleases NotI and XbaI. The duplex DNA is cloned into the mammalian expression cloning vector pcDNA3 (Invitrogen, CA). Expression of the ribozyme is under the control of the CMV promoter present in pcDNA3. The transcripts consist of approximately 140 nucleotides both 5' and 3' to the ribozyme sequence. The expression level of the transcribed sequence is ascertained by Northern blot analysis. If the RNA level is low additional sequences will be included in the construct in order to generate a longer and more stable transcript.

The construct is transfected using the electroporation technique into porcine primary endothelial cells, porcine B cells (L231). Also, the vector is co-transfected with plasmid expressing the porcine  $\alpha(1,3)$  galactosyltransferase into COS7 cells. Since COS7 cells do not express an endogenous  $\alpha(1,3)$  galactosyltransferase, the effect of the presence of the ribozyme on the expression of the introduced porcine  $\alpha(1,3)$  galactosyltransferase gene is easily ascertained.

#### EXAMPLE 5

#### TRANSGENIC SWINE PRODUCING ANTISENSE OR RIBOZYME RNA THAT INHIBIT $\alpha(1,3)$ GALACTOSYLTRANSFERASE SYNTHESIS

This approach requires direct microinjection of transgene DNA into one of the pronuclei of a porcine embryo

at the zygote stage (one-cell embryo). Injected one-cell embryos are transferred to recipient foster gilts for development to term (Hammer et al. 1985, Nature 315:680; Pursel et al. 1989, Science 244:1281)

Critical to successfully accomplishing this approach is the age and weight of the donor pigs, preferentially the haplotype specific mini-swine. Optimally, the animals are of age 8 to 10 months and weigh 70 to 85 lbs. This increases the probability of obtaining adequate supply of one-cell embryos for microinjection of the transgenes. In order to allow for accurate timing of the embryo collections at that stage from a number of embryo donors, the gilts are synchronized using a preparation of synthetic progesterone (Regumate). Hormone implants are applied to designated gilts 30 days prior to the date of embryo collection. Twenty days later, ten days prior to the date of collection, the implants are removed and the animals are treated with additional hormones to induce superovulation i.e. to increase the number of embryos for microinjection. Three days following implant removal, the animals are treated with 400 to 1000 IU of pregnant mare serum gonadotropin (PMSG) and with 750 IU of human chorionic gonadotropin (hCG) three to four days later. These animals are bred by artificial insemination (AI) on two consecutive days following injection of hCG.

Embryo collections are performed as follows: three days following the initial injection of hCG, the animals are anesthetized with an intramuscular injection of Telazol (3 mg/lb.), Rompum (2 mg/lb.) and Atropine (1 mg/lb.). A midline laparotomy is performed and the reproductive tract exteriorized. Collection of the zygotes is performed by cannulating the ampulla of the oviduct and flushing the oviduct with 10 to 15 ml phosphate buffered saline, prewarmed to 39° C. Following the collection, the donor animals are



prepared for recovery from surgery according to USDA guidelines. Animals used twice for embryo collections are euthanized according to USDA guidelines.

Injection of the transgene DNA into the pronuclei of the zygotes is carried out as follows. Zygotes are maintained in HAM's F-12 medium supplemented with 10% fetal calf serum at 38° C in 5 % CO<sub>2</sub> atmosphere. For injection the zygotes are placed into modified BMOC-2 medium containing HEPES salts (Ebert et al. 1984. J. Embryol. Exp. Morph. 84:91-103), centrifuged at 13,000 x g to partition the embryonic lipids and visualize the pronuclei. The embryos are placed in an injection chamber (depression slide) containing the same medium overlaid with light paraffin oil. Microinjection is performed on a Nikon Diaphot inverted-microscope equipped with Nomarski optics and Narishige micro-manipulators. Using 40x lens power the embryos are held in place with a holding pipette and injected with a glass needle which is back-filled with the solution of DNA containing the transgene (2 g/ml). Injection of approximately 2 picoliters of the solution (4 femtograms of DNA) which is equivalent to around 500 copies of the transgene is monitored by the swelling of the pronucleus by about 50%. Embryos that are injected are placed into the incubator prior to transfer to recipient animals.

Recipient animals are prepared similar to the donor animals, but not superovulated. Prior to the transfer of the injected embryos, recipient gilts are anesthetized, the abdomen opened surgically by applying a longitudinal incision and the ovaries exteriorized. The oviduct ipsilateral to the ovary with the larger number of corpus lutei is flushed, the embryos checked to evaluate if the animals is reproductively sound. Approximately 4 to 6 zygotes injected with the transgene are transferred to the flushed oviduct, the

abdominal incision sutured and the animals placed in a warm area for recovery. The status of the pregnancy is monitored by ultrasound starting at day 25, or approximately one week following the expected date of implantation. Pregnant recipients are housed separately until they are due to farrow.

Newborn piglets are analyzed for integration of the transgene into chromosomal DNA. Genomic DNA is extracted from an ear punch or a blood sample and initial screening is performed using PCR. Animals that are potentially transgene-positive are confirmed by Southern analysis. Transgenic founder animals are subjected to further analysis including the levels of expression, phenotype and germ line transmission. Northern analysis from RNA of selective tissues including endothelial cells is performed to determine the level of transgene expression. Also, immunological assays including flow cytometric analysis for binding of antibody from human serum and complement mediated lysis of pig cells recognized by human natural antibodies are carried out to evaluate the transgene effect. Animals that satisfy the above criteria are used as founders for breeding of a transgenic line. If the founder transgenic animals only satisfy part of the requirements, breeding and specific intercrossing of transgenic offspring is performed to assay the transgene effect in homozygous animals.

#### EXAMPLE 6

##### A SWINE MADE NULL-MUTANT FOR $\alpha(1,3)$

##### GALACTOSYLTRANSFERASE BY HOMOLOGOUS RECOMBINATION USING PORCINE EMBRYONIC STEM CELLS

Gene targeting by homologous recombination in swine requires several components, including the following: (A) a mutant gene targeting construct including the

positive/negative drug-selectable marker genes (Tubulewicz et al. 1991. Cell 65:1153); (B) embryonic stem cell cultures; and (C) the experimental embryology to reconstitute an animal from the cultured cells.

The targeting construct is provided from a genomic clone that spans most of the  $\alpha(1,3)$  galactosyltransferase gene and is isolated from a library made of isogenic DNA from a major histocompatibility complex (MHC) haplotype d/d of the miniature swine. Fragments of that genomic clone are introduced into a positive/negative selectable marker cassette specifically developed for gene targeting in embryonic stem (ES) cells and termed pPNT (Tubulewicz et al. 1991. Cell 65:1153). This gene targeting cassette includes as positive selectable marker the bacterial neomycin phosphotransferase gene (*neo*) which allows for selection of cells in G418. The *neo* gene is regulated by a promoter that guarantees high level expression in ES cells such as the phosphoglycerate kinase promoter-1 (PGK-1). Negative selection is accomplished by expressing the Herpes Simplex Virus - thymidine kinase (HSV-tk) gene which allows for selective killing of cells in Gancyclovir. Similar to the *neo* gene, the HSV-tk gene is regulated by the PGK-1 promoter, as well. In the targeting cassette pPNT there are unique and convenient cloning sites between the *neo* and the HSV-tk gene which are suitable sites to introduce the genomic fragment of the  $\alpha(1,3)$  galactosyltransferase gene upstream of the translation initiation signal AUG (e.g. SalI sites in introns 2 and 4) This fragment of approximately 2 kb of DNA is cloned in reverse orientation to the direction of transcription of the PGK-*neo* cassette to assure that no truncated or residual peptide is generated at the  $\alpha(1,3)$  galactosyltransferase-locus. Genomic sequences of the  $\alpha(1,3)$  galactosyltransferase locus downstream of exon 4, approximately 5 kb are introduced into pPNT at the 5'-end of the *neo* gene. This targeting

construction termed pPNT-alpha GT1 is linearized and transfected by electroporation into porcine ES cells. Double selection in G418 (150 to 300  $\mu$ g/ml) and Gancyclovir is performed to initially isolate clones of ES cells with targeted mutations in the  $\alpha(1,3)$  galactosyltransferase locus. Confirmation of homologous recombinant clones is achieved using Southern analysis.

ES cell clones that have undergone targeted mutagenesis of one allele of the  $\alpha(1,3)$  galactosyltransferase locus are subjected to a second round of *in vitro* mutagenesis or used for reconstituting an animal that contains the mutation. A second round of *in vitro* mutagenesis can be carried out using an analogous targeting construction with hygromycin phosphotransferase *hyg* as positive selectable marker gene.

As far as the reconstitution of animals is concerned, the methods include nuclear transfer, blastocyst injection or morula aggregation. The preferred routes include either blastocyst injection or morula aggregation which yield chimeras between the donor cells and the recipient embryos. For both these methods recipient embryos are prepared as follows: embryo donor/recipient gilts are synchronized and mated as described in Example 5. On day 6 following artificial insemination or natural mating, the gilts are prepared for surgery as described earlier, anesthetized and the uteri retrogradely flushed using a prewarmed (38° C) solution of phosphate buffered saline (PBS). Intact blastocysts that are encapsulated by the *zona pellucida* are placed in a depression slide containing HEPES-buffered medium (Whitten's or TL-HEPES) and approximately 15 to 20 ES cells are injected into the blastocoel using a glass injection needle with an opening of 20  $\mu$ m and Narishige micromanipulators. Injected embryos are then reimplanted into recipient foster gilts for development to term and

pregnancies are monitored using ultrasound. Offspring is analyzed for chimerism using the polymerase chain reaction (PCR) of DNA samples extracted from blood, skin and tissue biopsies and primers complementary to the *neo* or *hyg* gene. Germ line transmission of the chimeras is assayed using PCR and *in situ* hybridization of tissue samples obtained from male and female gonads. Male and female chimeras which transmit the ES cell genotype to the germ line are crossed to yield homozygously mutant animals. Analysis of mutant animals for expression of  $\alpha(1,3)$  galactosyltransferase and binding of human natural antibodies to endothelial cells of those animals is used as final test to assess the validity of gene knock out approach in swine.

#### EXAMPLE 7

#### A SWINE MADE NULL-MUTANT FOR $\alpha(1,3)$ GALACTOSYLTRANSFERASE USING ISOGENIC DNA TARGETING INTO ZYGOTES

Isogenic DNA or DNA that is substantially identical in sequence between the targeting vector and target DNA in the chromosomes greatly increases the frequency for homologous recombination events and thus the gene targeting efficiency. Using isogenic DNA-targeting vectors, targeting frequencies of 80% or higher are achieved in mouse embryonic stem cells. In contrast, non-isogenic DNA-vectors normally yield targeting frequencies of around 0.5% to 5%, i.e., approximately two orders of magnitude lower than isogenic DNA vectors. Surprisingly, isogenic DNA constructions are predominantly integrated into chromosomes by homologous recombination rather than random integration. As a consequence, targeted mutagenesis of genes can, therefore, be carried out in biological systems, including animals and even zygotes, which do not lend themselves to the use of elaborate selection protocols.

The significance for isogenic DNA in gene targeting approaches was first described by TerRiele *et al.*, PNAS 89, 5128-5132 (1992) and also in PCT/US 92/07184.

In order for the isogenic DNA approach to be feasible, targeting vectors have to be constructed from a source of DNA that is identical to the DNA of the organism to be targeted. Ideally, isogenic DNA targeting is carried out in inbred strains of animals in which all genetic loci are homozygous. All animals of that strain can therefore serve as a source for generating isogenic targeting vectors. In cases in which inbred strains of animals are lacking, genetic loci can consist of many different alleles. Thus, isogenic vectors can only be derived from the individual in which targeted gene replacement is attempted. Furthermore, the targeting vector would be isogenic for one allele only.

In the haplotype specific miniature swine, isogenic gene targeting vectors are readily derived for the MHC locus, since that genomic region has been maintained homozygous for several generations. Since the cDNA sequence to  $\alpha$  (1,3) galactosyltransferase locus has been determined (Example 1) and genomic clones have been generated recently it is now possible to map and thereby avoid, polymorphisms within the  $\alpha$  (1,3) galactosyltransferase locus gene.

Mapping of polymorphism is carried out as follows. A group of mini-swine that are closely related, either siblings and/or parent/offspring is identified. High molecular weight DNA is extracted and PCR reactions are set up to amplify sequences from Exon 6 to Exon 7 spanning Intron 6. The organization of Exons 5 through 9 of the porcine gene are known to be homologous to those of the murine gene (Figure 4, Joziassse, D.H., Shaper, N.L., Kim, D., Van den Eijnden, D. and Joel H. Shaper, J. Biol. Chem. 167, pp. 5534-5541

(1992)). In Figure 4, genomic organization of the murine  $\alpha$  (1,3-) galactosyltransferase gene Exons are labeled 1-9 and are indicated by solid boxes. Intron sequences are represented by a thin line. The 5'-oligo in Exon 6 has the following sequence: 5'- TCC GAG CTG GTT TAA CAA TGG GTA -3' (SEQ ID NO: 25) and the 3' oligo in Exon 7 contains the following sequence: 5'- TCT TCG TGG TAA CTG TGA GTC CTA -3' (SEQ ID NO: 26). The PCR fragments are approximately 1 and 2 kb long. In order to assay for PCR-RFLPs, restriction digestions using 4-cutters are carried out which are expected to cut frequently. Such 4-cutters include BstU I (CGCG), Hae III (GGCC), Hha I (GCGC) Sau3A (GATC), Rsa I (GTAC) and Taq I (TCGA), which are commercially available from New England BioLabs (Beverly, MA). Based on the pattern of restriction fragments obtained from parental animals and offspring, as well as between siblings, it is possible to define the number of different alleles present in that group of animals. In case the PCR-RFLP does not yield obvious sequence polymorphisms between different alleles in different animals, alternative methods can be employed including DNA sequencing or enzyme mismatch cleavage (EMC) using T7 or T4 resolvases. Additional animals are then analyzed in a similar manner to identify specific alleles at the  $\alpha$  1,3 galactosyltransferase locus. Once several alleles have been identified, targeting constructions are prepared for those alleles that are most abundantly present in the line of mini-swine selected for zygote injection.

Gene targeting constructions are generated as follows. A genomic library is made in lambda replacement vectors including Lambda FIX II, Lambda EMBL4 or Lambda Dash II (Stratagene Cloning Systems). The library is then plated at a density of around 50,000 plaques/15 cm plate and screened with a probe specific for Exon 9 of the swine  $\alpha$  (1,3) galactosyltransferase gene. The probe is generated using PCR

which is carried out using a standard protocol: 35 cycles, anneal at 60°C for 20 sec, extend at 72°C for 40 sec and denature at 94°C for 20 sec. Positive clones with insert sizes of around 15 kb are then subcloned into plasmid vectors including pSP72 and engineered for targeting as either an insertion vector or a replacement vector.

An insertion vector is designed as described by Hasty and Bradley (Gene Targeting Vectors for Mammalian Cells, in Gene Targeting: A Practical Approach, ed, Alexandra L. Joyner, IRL Press 1993). Insertion vectors require for only one crossover to occur for integration by homologous recombination into the native locus. The double strand breaks, the two ends of the vector which are known to be highly recombinogenic, are located on adjacent sequences on the chromosome. Preferably the site of integration of the insertion vector is at the beginning of Intron 8 of the  $\alpha$  (1,3) galactosyltransferase gene. The entire length of Intron 8 is thereby included as a homologous and identical sequence, carrying the minimal number mismatches. Exon 9 which encodes the catalytic domain of the enzyme is then substituted by a selectable marker such as the neomycin phosphotransferase gene, or a derivative thereof termed IRES  $\delta$ geo. Downstream, or 3' of the selectable marker, sequences from Intron 7, Exon 8 and Intron 8 are included, terminating at the site adjacent to the beginning of the vector. The targeting frequencies of that construction are in the range of 30 to 50%. All these constructions are made using standard cloning procedures.

The replacement vectors have also been extensively described by Hasty and Bradley, supra. Conceptually more straightforward than the insertion vector, replacement vectors issued here are essentially co-linear fragments of a certain stretch of genomic sequence at the  $\alpha$  (1,3)



galactosyltransferase locus. Preferably, the DNA sequence from which an isogenic replacement vector is constructed include approximately 6 to 10 kb of uninterrupted DNA. This DNA corresponds to a region between the start of Exon 4 and the end of Intron 8 of certain alleles of the  $\alpha$  (1,3) galactosyltransferase locus from mini-swine. Exon 9, encoding the catalytic domain is replaced with the selectable marker gene, such as the neo gene, or a functional analog thereof. The 3'-end of the replacement vector includes the 3' untranslated region of the gene in Exon 9, including approximately 1kb of isogenic DNA. Two crossovers, one on either side of the selectable marker causes the mutant targeting vector to become integrated and replace the wild-type gene.

Microinjection of the isogenic transgene DNA into one of the pronuclei of a porcine embryo at the zygote stage (one-cell embryo), particularly the mini-swine, is accomplished by modification of the protocol described earlier. (Hammer et al. 1985, Nature 315, 680; Pursel et al 1989, Science 244, 1281). Optimally, the mini-swine are of age 8 to 10 months and weigh 70 to 85 lbs. This increases the probability of obtaining an adequate supply of one-cell embryo donors. The gilts are synchronized using a preparation of synthetic progesterone (Regumate). Hormone implants are applied to designated gilts 30 days prior to the date of embryo collection. Twenty days later, ten days prior to the date of collection, the implants are removed and the animals are treated with additional hormones to induce superovulation, i.e., to increase the number of embryos for microinjection. Three days following implant removal, the animals are treated with 400 to 1000 IU of pregnant mare serum gonadotropin (PMSG) and with 750 IU of human chorionic gonadotropin (hCG) three to four days later. These animals are bred by

artificial insemination (AI) on two consecutive days following injection of hCG.

Embryo collections are performed as follows. Three days following the initial injection of hCG, the animals are anesthetized with an intramuscular injection of Telazol (3 mg/lb), Rompum (2 mg/lb) and Atropine (1 mg/lb). A midline laparotomy is performed and the reproductive tract exteriorized. Collection of the zygotes is performed by cannulating the ampulla of the oviduct and flushing the oviduct with 10 to 15 ml phosphate buffered saline, prewarmed to 39°C. Following the collection, the donor animals are prepared for recovery from surgery according to USDA guidelines. Animals used twice for embryo collections are euthanized according to USDA guidelines.

Injection of the transgene DNA into the pronuclei of the zygotes is carried out as summarized below. Zygotes are maintained in HAM F-12 medium supplemented with 10% fetal calf serum at 38°C in 5% CO<sub>2</sub> atmosphere. For injection the zygotes are placed into BMOC-2 medium, centrifuged at 13,000 g to partition the embryonic lipids and visualize the pronuclei. The embryos are placed in an injection chamber (depression slide) containing the same medium overlaid with light paraffin oil. Microinjection is performed on a Nikon Diaphot inverted-microscope equipped with Nomarski optics and Narishige micromanipulators. Using 40x lens power the embryos are held in place with a holding pipette and injected with a glass needle which is back-filled with the solution of DNA containing the transgene (2 µg/ml). Injection of approximately 2 picoliters of the solution (4 femtograms of DNA) which is equivalent to around 500 copies of the transgene is monitored by the swelling of the pronucleus by about 50%. Embryos that are injected are placed into the incubator prior to transfer to recipient animals.

Recipient animals are prepared similar to the donor animals, but not superovulated. Prior to the transfer of the injected embryos, recipient gilts are anesthetized, the abdomen opened surgically by applying a longitudinal incision and the ovaries exteriorized. The oviduct ipsilateral to the ovary with the larger number of corpus lutei is flushed and the embryos are checked to evaluate if the animal is reproductively sound. Approximately 4 to 6 zygotes injected with the transgene are transferred to the flushed oviduct, the abdominal incision sutured and the animals placed in a warm area for recovery. The status of the pregnancy is monitored by ultrasound starting at day 25, or approximately one week following the expected date of implantation. Pregnant recipients are housed separately until they are due to farrow.

Newborn piglets are analyzed for integration of the transgene into chromosomal DNA. Genomic DNA is extracted from an ear punch or a blood sample and initial screening is performed using PCR. Animals that are potentially transgene-positive are confirmed by Southern analysis. Transgenic founder animals are subjected to further analysis regarding the locus of transgene integration using Southern analysis. Those animals that have the transgene targeted to the native  $\alpha$  (1,3) galactosyltransferase locus, i.e., that are heterozygous mutant for that gene, are grown up and bred to additional heterozygous mutant founder animals. Applying Mendelian genetics allows us to breed animals that are homozygous mutant for the  $\alpha$  (1,3) galactosyltransferase. Functional assays using the lectin BI4 and human preformed natural antibodies are then used to look at expression of the Gal  $\alpha$  (1,3) Gal epitope on swine cells and to confirm the knockout of the  $\alpha$  (1,3) galactosyltransferase gene.

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT(S): BIOTRANSPLANT, INC.  
THE GENERAL HOSPITAL CORPORATION  
THE UNIVERSITY OF LONDON  
BAETSCHER, Manfred W.  
GUSTAFSSON, Kenth T.  
SACHS, David. H.
- (ii) TITLE OF INVENTION:  $\alpha$ (1,3) GALACTOSYLTRANSFERASE NEGATIVE SWINE
- (iii) NUMBER OF SEQUENCES: 26
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Carella, Byrne, Bain, Gilfillan, Cecchi,  
Stewart & Olstein
- (B) STREET: 6 Becker Farm Road
- (C) CITY: Roseland
- (D) STATE: New Jersey
- (E) COUNTRY: USA
- (F) ZIP: 07068
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5 inch diskette
- (B) COMPUTER: IBM PS/2
- (C) OPERATING SYSTEM: MS-DOS
- (D) SOFTWARE: ASCII
- (vi) PARENT APPLICATION DATA:
- APPLICATION NUMBER: 08/228,933
- FILING DATE: April 13, 1994
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Charles J. Herron
- (B) REGISTRATION NUMBER: 28,019
- (C) REFERENCE/DOCKET NUMBER: 61750-CIP

## (2) INFORMATION FOR SEQ ID NO: 1

- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 1269 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (iii) HYPOTHETICAL: NO

(iv) ANTISENSE:

NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

CATGAGGAGA AAATA ATG AAT GTC AAA GGA AGA GTG GTT CTG TCA ATG	48
Met Asn Val Lys Gly Arg Val Val Leu Ser Met	
1 5 10	
CTG CTT GTC TCA ACT GTA ATG GTT GTG TTT TGG GAA TAC ATC AAC AGC	96
Leu Leu Val Ser Thr Val Met Val Val Phe Trp Glu Tyr Ile Asn Ser	
15 20 25	
CCA GAA GGT TCT TTG TTC TGG ATA TAC CAG TCA AAA AAC CCA GAA GTT	144
Pro Glu Gly Ser Leu Phe Trp Ile Tyr Gln Ser Lys Asn Pro Glu Val	
30 35 40	
GGC AGC AGT GCT CAG AGG GGC TGG TGG TTT CCG AGC TGG TTT AAC AAT	192
Gly Ser Ser Ala Gln Arg Gly Trp Trp Phe Pro Ser Trp Phe Asn Asn	
45 50 55	
GGG ACT CAC AGT TAC CAC GAA GAA GAA GAC GCT ATA GGC AAC GAA AAG	240
Gly Thr His Ser Tyr His Glu Glu Glu Asp Ala Ile Gly Asn Glu Lys	
60 65 70 75	
GAA CAA AGA AAA GAA GAC AAC AGA GGA GAG CTT CCG CTA GTG GAC TGG	288
Glu Gln Arg Lys Glu Asp Asn Arg Gly Glu Leu Pro Leu Val Asp Trp	
80 85 90	
TTT AAT CCT GAG AAA CGC CCA GAG GTC GTG ACC ATA ACC AGA TGG AAG	336
Phe Asn Pro Glu Lys Arg Pro Glu Val Val Thr Ile Thr Arg Trp Lys	
95 100 105	
GCT CCA GTG GTA TGG GAA GGC ACT TAC AAC AGA GCC GTC TTA GAT AAT	384
Ala Pro Val Val Trp Glu Gly Thr Tyr Asn Arg Ala Val Leu Asp Asn	
110 115 120	
TAT TAT GCC AAA CAG AAA ATT ACC GTG GGC TTG ACG GTT TTT GCT GTC	432
Tyr Tyr Ala Lys Gln Lys Ile Thr Val Gly Leu Thr Val Phe Ala Val	
125 130 135	
GGA AGA TAC ATT GAG CAT TAC TTG GAG GAG TTC TTA ATA TCT GCA AAT	480
Gly Arg Tyr Ile Glu His Tyr Leu Glu Glu Phe Leu Ile Ser Ala Asn	
140 145 150 155	

ACA TAC TTC ATG GTT GGC CAC AAA GTC ATC TTT TAC ATC ATG GTG GAT Thr Tyr Phe Met Val Gly His Lys Val Ile Phe Tyr Ile Met Val Asp 160 165 170	528
GAT ATC TCC AGG ATG CCT TTG ATA GAG CTG GGT CCT CTG CGT TCC TTT Asp Ile Ser Arg Met Pro Leu Ile Glu Leu Gly Pro Leu Arg Ser Phe 175 180 185	576
AAA GTG TTT GAG ATC AAG TCC GAG AAG AGG TGG CAA GAC ATC AGC ATG Lys Val Phe Glu Ile Lys Ser Glu Lys Arg Trp Gln Asp Ile Ser Met 190 195 200	624
ATG CGC ATG AAG ACC ATC GGG GAG CAC ATC CTG GCC CAC ATC CAG CAC Met Arg Met Lys Thr Ile Gly Glu His Ile Leu Ala His Ile Gln His 205 210 215	672
GAG GTG GAC TTC CTC TTC TGC ATG GAC GTG GAT CAG GTC TTC CAA AAC Glu Val Asp Phe Leu Phe Cys Met Asp Val Asp Gln Val Phe Gln Asn 220 225 230 235	720
AAC TTT GGG GTG GAG ACC CTG GGC CAG TCG GTG GCT CAG CTA CAG GCC Asn Phe Gly Val Glu Thr Leu Gly Gln Ser Val Ala Gln Leu Gln Ala 240 245 250	768
TGG TGG TAC AAG GCA CAT CCT GAC GAG TTC ACC TAC GAG AGG CGG AAG Trp Trp Tyr Lys Ala His Pro Asp Glu Phe Thr Tyr Glu Arg Arg Lys 255 260 265	816
GAG TCC GCA GCC TAC ATT CCG TTT GGC CAG GGG GAT TTT TAT TAC CAC Glu Ser Ala Ala Tyr Ile Pro Phe Gly Gln Gly Asp Phe Tyr Tyr His 270 275 280	864
GCA GCC ATT TTT GGG GGA ACA CCC ACT CAG GTT CTA AAC ATC ACT CAG Ala Ala Ile Phe Gly Gly Thr Pro Thr Gln Val Leu Asn Ile Thr Gln 285 290 295	912
GAG TGC TTC AAG GGA ATC CTC CAG GAC AAG GAA AAT GAC ATA GAA GCC Glu Cys Phe Lys Gly Ile Leu Gln Asp Lys Glu Asn Asp Ile Glu Ala 300 305 310 315	960
GAT TGG CAT GAT GAA AGC CAT CTA AAC AAG TAT TTC CTT CTC AAC AAA G Trp His Asp Glu Ser His Leu Asn Lys Tyr Phe Leu Leu Asn Lys 320 325 330	1008

CCC ACT AAA ATC TTA TCC CCA GAA TAC TGC TGG GAT TAT CAT ATA GGC 1056  
 Pro Thr Lys Ile Leu Ser Pro Glu Tyr Cys Trp Asp Tyr His Ile Gly  
                   335                                  340                                  345

ATG TCT GTG GAT ATT AGG ATT GTC AAG ATA GCT TGG CAG AAA AAA GAG 1104  
 Met Ser Val Asp Ile Arg Ile Val Lys Ile Ala Trp Gln Lys Lys Glu  
                   350                                  355                                  360

TAT AAT TTG GTT AGA AAT AAC ATC TGA CTTTAAATTG TGCCAGCAGT 1151  
 Tyr Asn Leu Val Arg Asn Asn Ile  
                   365                                  370

TTTCTGAATT TGAAAGAGTA TTACTCTGGC TACTTCCTCA GAGAAGTAGC 1201  
 ACTTAATTTT AACTTTTAAA AAAATACTAA CAAAATACCA ACACAGTAAG 1251  
 TACATATTAT TCTTCCTT 1269

## (3) INFORMATION FOR SEQ ID NO: 2

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

AAGCTTAAGC TATCGGAC

18

## (4) INFORMATION FOR SEQ ID NO: 3

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: YES  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

CTTAATATCC GCAGGTAG

18

## (5) INFORMATION FOR SEQ ID NO: 4

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

CGCTCTAGAA CTAGTGGATC

20

## (6) INFORMATION FOR SEQ ID NO: 5

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: YES  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5:

CAAAGAACCT TCTGGGCT

18



(7) INFORMATION FOR SEQ ID NO: 6  
 (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 19 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 6:

GGCTGGTGGT TTCCGAGC

19

(8) INFORMATION FOR SEQ ID NO: 7  
 (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 7:

CTCCAAGTAA TCGTCAAT

18

(9) INFORMATION FOR SEQ ID NO: 8  
 (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 8:

AGGATGCCTT TGATAGAG

18

(10) INFORMATION FOR SEQ ID NO: 9  
 (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 9:

GTCTTGCCAC CTCTTCTCGG

20

## (11) INFORMATION FOR SEQ ID NO: 10

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 10:

TTCCTCTTCT GCATGGAC

18

## (12) INFORMATION FOR SEQ ID NO: 11

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 11:

GGGGATTTTT ATTACCAC

18

## (13) INFORMATION FOR SEQ ID NO: 12

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 12:

GATGAAAGCC ATCTAAAC

18

- (14) INFORMATION FOR SEQ ID NO: 13
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 20 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 13:  
ACTCTTCCTT TGACATTCAT
- 20
- (15) INFORMATION FOR SEQ ID NO: 14
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 23 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 14:  
AGCAGCATTG ACAGAACCAC TCT
- 23
- (16) INFORMATION FOR SEQ ID NO: 15
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 18 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 15:  
ACATTCATTA TTTTCTCC
- 18
- (17) INFORMATION FOR SEQ ID NO: 16
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 57 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 16:

GGCCGCTCTG GTTATGGTCA CCTGATGAGT CCGTGAGGAC GAAACCTCTG GGCCTTT

57

## (18) INFORMATION FOR SEQ ID NO: 17

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 36 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (iii) HYPOTHETICAL:

NO

## (iv) ANTISENSE:

NO

## (xi) SEQUENCE DESCRIPTION:

SEQ ID NO. 17:

CTAGAAACGC CCAGAGGTCG TGACCATAAC CAGAGC

36

## (19) INFORMATION FOR SEQ ID NO: 18

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 57 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (iii) HYPOTHETICAL:

NO

## (iv) ANTISENSE:

YES

## (xi) SEQUENCE DESCRIPTION:

SEQ ID NO. 18:

GGCCGCCTCA ATGTATCTTC CCTGATGAGT CCGTGAGGAC GAAACAGCAA AAACCGT

57

## (120) INFORMATION FOR SEQ ID NO: 19

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 36 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (iii) HYPOTHETICAL:

NO

## (iv) ANTISENSE:

NO

## (xi) SEQUENCE DESCRIPTION:

SEQ ID NO. 19:

CTAGACGGTT TTTGCTGTCG GAAGATACAT TGAGGC

36

## (21) INFORMATION FOR SEQ ID NO: 20

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 58 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (iii) HYPOTHETICAL:

NO

## (iv) ANTISENSE:

YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 20:  
 GGCCGCAAAG GAACGCAGAG CTGATGAGTC CGTGAGGACG AAACCCAGCT CTATCAAT

58

(22) INFORMATION FOR SEQ ID NO: 21

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 37 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 21:  
 CTAGATTGAT AGAGCTGGGT CCTCTGCGTT CCTTTGC

37

(23) INFORMATION FOR SEQ ID NO: 22

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 51 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: Linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 22 :  
 UCUGGUUAUG GUCACCUGAU GAGUCCGUGA GGACGAAACC UCUGGGCGUU U

51

(24) INFORMATION FOR SEQ ID NO: 23

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 51 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 23 :  
 CUCAAUGUAU CUUCCUGAU GAGUCCGUGA GGACGAAACA GCAAAAACCG U

51

## (25) INFORMATION FOR SEQ ID NO: 24

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 51 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 24 :

AAAGGAACGC AGAGCUGAUG AGUCCGUGAG GACGAAACCC AGCUCUAUCA A

51

## (26) INFORMATION FOR SEQ ID NO: 25

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 25 :

TCCGAGCTGG TTAAACAATG GGTA

24

## (27) INFORMATION FOR SEQ ID NO: 26

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 26 :

TCITCGTGGT AACTGTGATG CCTA

24

## WHAT IS CLAIMED IS:

1. A transgenic swine in which the normal expression of the  $\alpha(1,3)$  galactosyltransferase is prevented in at least one organ or tissue type.
2. The transgenic swine of claim 1 which has been made to produce a nucleic acid sequence that binds to and prevents the translation of mRNA coding for  $\alpha(1,3)$  galactosyltransferase in said swine.
3. The transgenic swine of claim 2 whose genome had been modified to include a construct comprising a DNA encoding an antisense RNA to that portion of the  $\alpha(1,3)$  galactosyltransferase coding region necessary to prevent or express a functional enzyme operably linked to a promoter therefore.
4. The transgenic swine of claim 3 wherein the promoter is a strong non-tissue specific constitutive or regulatable promoter.
5. The transgenic swine of claim 3 wherein the promoter is a strong tissue specific constitutive or regulatable promoter.
6. The transgenic swine of claim 1 which has been made to produce ribozyme.
7. The transgenic swine of claim 6 which has been modified to include a construct comprising DNA coding for at least that portion of a ribozyme necessary to inactivate  $\alpha(1,3)$  galactosyltransferase operably linked to promoter therefor.

8. The transgenic swine of claim 7 wherein the promoter is a strong non-tissue specific constitutive or regulatable promoter.

9. The transgenic swine of claim 7 wherein the promoter is a strong tissue specific constitutive or regulatable promoter.

10. An  $\alpha(1,3)$  galactosyltransferase negative swine grown from a porcine oocyte whose pronuclear material has been removed and into which has been introduced a pluripotent porcine embryonic stemcell that is negative for the expression of  $\alpha(1,3)$  galactosyltransferase

11. A nucleic acid having a sequence selected from the group consisting of SEQ. ID. NOS. 1 and 13 through 24.

12. The nucleic acid of claim 11 having the sequence set forth in SEQ. ID. NO. 1.

13. The nucleic acid of claim 11 wherein the SEQ. ID. NOS. are selected from the group consisting of SEQ. ID. NOS. 13 through 15.

14. The nucleic acid of claim 11 wherein the SEQ. ID. NOS. are selected from the group consisting of SEQ. ID. NOS. 16 through 21.

15. The nucleic acid of claim 11 wherein the SEQ. ID. NOS. are selected from the group consisting of SEQ. ID. NOS. 22 through 24.

16. The transgenic swine of Claim 1 in which the normal expression of the  $\beta(1,3)$  galactosyltransferase gene is



prevented by introduction to said swine of an isogenic DNA-containing targeting vector.

17. The  $\alpha$  (1,3) galactosyltransferase negative swine of Claim 16 grown from a porcine zygote which has been modified by homologous recombination with an isogenic DNA targeting vector at the  $\alpha$  (1,3) galactosyltransferase chromosomal locus.

18. The  $\alpha$  (1,3) galactosyltransferase negative swine of Claim 17 wherein the isogenic DNA targeting vector comprises at least about 6 kb of isogenic DNA and a promoterless selectable marker gene.

# FIG. 1A

5'-CATGAGGAGA AAATA ATG AAT GTC AAA GGA AGA GTG GTT CTG TCA ATG  
 CTG CTT GTC TCA ACT GTA ATG GTT GTG TTT TGG GAA TAC ATC AAC AGC  
 CCA GAA GGT TCT TTG TTC TGG ATA TAC CAG TCA AAA AAC CCA GAA GTT  
 GGC AGC AGT GCT CAG AGG GGC TGG TGG TTT CCG AGC TGG TTT AAC AAT  
 GGG ACT CAC AGT TAC CAC GAA GAA GAA GAC GCT ATA GGC AAC GAA AAG  
 GAA CAA AGA AAA GAA GAA GAC AAC AGA GGA GAG CTT CCG CTA GTG GAC TGG  
 TTT AAT CCT GAG AAA CCG CCA GAG GTC GTG ACC ATA ACC AGA TGG AAG  
 GCT CCA GTG GTA TGG GAA GGC ACT TAC AAC AGA GCC GTC TTA GAT AAT  
 TAT TAT GCC AAA CAG AAA ATT ACC GTG GGC TTG ACG GTT TTT GCT GTC  
 GGA AGA TAC ATT GAG CAT TAC TTG GAG GAG TTC TTA ATA TCT GCA AAT  
 ACA TAC TTC ATG GTT GGC CAC AAA GTC ATC TTT TAC ATC ATG GTG GAT  
 GAT ATC TCC AGG ATG CCT TTG ATA GAG CTG GGT CCT CTG CGT TCC TTT  
 AAA GTG TTT GAG ATC AAG TCC GAG AAG AGG TGG CAA GAC ATC AGC ATG  
 ATG CGC ATG AAG ACC ATC GGG GAG CAC ATC CTG GCC CAC ATC CAG CAC

MATCH WITH FIG. 1B

## FIG. 1B

MATCH WITH FIG. 1A

GAG GTG GAC TTC CTC TTC TGC ATG GAC GTG GAT CAG GTC TTC CAA AAC  
 AAC TTT GGG GTG GAG ACC CTG GGC CAG TCG GTG GCT CAG CTA CAG GCC  
 TGG TGG TAC AAG GCA CAT CCT GAC GAG TTC ACC TAC GAG AGG CGG AAG  
 GAG TCC GCA GCC TAC ATT CCG TTT GGC CAG GGG GAT TTT TAT TAC CAC  
 GCA GCC ATT TTT GGG GGA ACA CCC ACT CAG GTT CTA AAC ATC ACT CAG  
 GAG TGC TTC AAG GGA ATC CTC CAG GAC AAG GAA AAT GAC ATA GAA GCC  
 GAG TGG CAT GAT GAA AGC CAT CTA AAC AAG TAT TTC CTC AAC AAA  
 CCC ACT AAA ATC TTA TCC CCA GAA TAC TGC TGG GAT TAT CAT ATA GGC  
 ATG TCT GTG GAT ATT AGG ATT GTC AAG ATA GCT TGG CAG AAA AAA GAG  
 TAT AAT TTG GTT AGA AAT AAC ATC TGA CTTTAAATTG TGCCAGCAGT  
 TTTCTGAATT TGAAAGAGTA TTACTCTGGC TACTTCCTCA GAGAAAGTAGC ACTTAATTTT  
 AACTTTTAAA AAAATACTAA CAAATACCA ACACAGTAAG TACATATTAT TCTTCCTT -  
 3'

2/6

## FIG. 2A

Porcine	MNVKGRVVL	MLLVSTVMVV	FWEYINSPEG	SLFWIYQSKN	PEV-GSSAQR	49
Bovine	MNVKGKVIL	MLVVSTVIVV	FWEYIHSPEG	SLFWINPSRN	PEVGGSSIQK	50
Murine	MNVKGKVILL	MI.IVSTVVVV	FWEYVNSPDG	SFLWIYHTKI	PEVGENRWQK	50
Porcine	GWWFPSWFNN	GTHSYHEEED	AIGNEKEQRK	EDNRGELPLV	DWFNPEKRPE	99
Bovine	GWLPRWFNN	GYH---EEDG	DINEEKEQRN	EDESK-LKLS	DWFNPFKRPE	96
Murine	DWWFPSWFKN	GTHSYQ-EDN	VEGRREKGRN	GDRIEPQLW	DWFNPKNRPD	99
Porcine	VVTITRWKAP	VWEGTYNRA	VLDNYAKQK	ITVGLTVFAV	GRYIEHYLEE	149
Bovine	VVTMTKKWAP	VWEGTYNRA	VLDNYAKQK	ITVGLTVFAV	GRYIEHYLEE	146
Murine	VLTVTPWKAP	IVWEGTYDTA	LLEKYYATQK	LTVGLTVFAV	GKYIEHYLED	149
Porcine	FLISANTYFM	VGHKVIFYIM	VDDISRMPLI	ELGPLRSFKV	FEIKSEKRWQ	199
Bovine	FLTSANKHFM	VGHPVIFYIM	VDDVSRMPLI	ELGPLRSFKV	FKIKPEKRWQ	196
Murine	FLESADMYFM	VGHRVIFYVM	IDDTSRMPVV	HLNPLHSLQV	FEIRSEKRWQ	199

MATCH WITH FIG. 2B

## FIG. 2B

MATCH WITH FIG. 2B

Porcine	DISMMRMKTI	GEHILAHIQH	EVDFLFCMDV	DQVFQNNFGV	ETLGQSVAQL	249
Bovine	DISMMRMKTI	GEHIVAHIQH	EVDFLFCMDV	DQVFQDKFGV	ETLGESVAQL	246
Murine	DISMMRMKTI	GEHILAHIQH	EVDFLFCMDV	DQVFQDNFGV	ETLGQLVAQL	249
Porcine	QAWWYKAHPD	EFTYERRKES	AAIIPFGQGD	FYYHAAIFGG	TPTQVLNITQ	299
Bovine	QAWWYKADPN	DFTYERRKES	AAIIPFGEGD	FYYHAAIFGG	TPTQVLNITQ	296
Murine	QAWWYKASPE	KFTYERRELS	AAIIPFGEGD	FYYHAAIFGG	TPTHILNLTR	299
Porcine	ECFKGILQDK	ENDIEAEWHD	ESHLNKYFLL	NKPTKILSPE	YCWDYHIGMS	349
Bovine	ECFKGILKDK	KNDIEAQWHD	ESHLNKYFLL	NKPTKILSPE	YCWDYHIGLP	346
Murine	ECFKGILQDK	KHDIEAQWHD	ESHLNKYFLF	NKPTKILSPE	YCWDYQIGLP	349
Porcine	VDIRIVKIAW	QKKEYNLVRN	NI			371
Bovine	ADIKLVKMSW	QTKEYNVVRN	NV			368
Murine	SDIKSVKVAW	QTKEYNLVRN	NV			371

5' ——— GUCX ——— 3' mRNA

FIG. 3

consensus ribozyme cleavage site



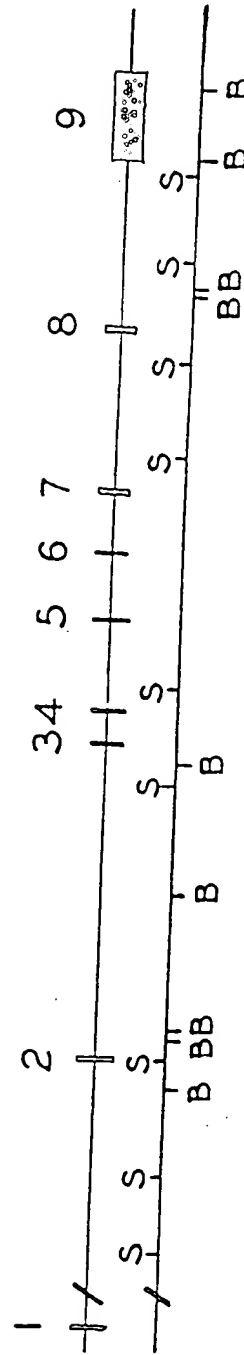
Stem I C Stem III  
 AAACGCCAGAGGU GUGACCAUAACCAGA A A  
 UUUGCGGGUCUCCA CACUGGUAUUGGUCU A A

A G C  
 G GGAC  
 T CCTC  
 G U

Stem II A G  
 G A  
 G A  
 T

## FIG. 4

Genomic Organization of the Mouse  
 $\alpha(1,3)$  Galactosyltransferase Gene



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03940

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 17/00; C12N 15/00, 15/09, 15/11, 15/52  
US CL : 435/172.3; 536/23.1, 24.1; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3; 536/23.1, 24.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases searched: APS; biosis, ca, capreviews, derwent, medline, uspatfull

Search terms: pig#; swine; transgen; antisens?; ribozym?; homologous; recombinat?; promoter?; galactosyl?; baetscher?/au; sachs?/au; gustafsson?/au

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IMMUNOLOGY LETTERS, Volume 29, issued 1991, T.D.H. Cairns et al., "Xenografts - future prospects for clinical transplantation", pages 167-170, see entire document.	1-5
Y	XENOGRAFT, Volume 25, issued 1989, R.L. Kirkman, "Of swine and men: Organ physiology in different species", pages 125-132, see entire document.	1-5
Y	JOURNAL OF REPRODUCTION AND FERTILITY, SUPPLEMENT, Volume 40, issued 1990, V.G. Pursel et al., "Expression and performance in transgenic pigs", pages 235-245, see entire document.	1-5

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:		T	
*A*	document defining the general state of the art which is not considered to be of particular relevance	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
*E*	earlier document published on or after the international filing date	X	
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
*O*	document referring to an oral disclosure, use, exhibition or other means	Y	
*P*	document published prior to the international filing date but later than the priority date claimed	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
		*G*	
		document member of the same patent family	

Date of the actual completion of the international search

23 MAY 1995

Date of mailing of the international search report

13.07.95

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03940

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL REVIEWS, Volume 90, number 4, issued June 1990, E. Uhlmann et al., "Antisense oligonucleotides: A new therapeutic principle", pages 543-584, see entire document.	1-5
Y	NATURE, Volume 355, issued 20 February 1992, M.C. Pepin et al., "Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene", pages 725-728, see entire document.	1-5
Y	SOMATIC CELL AND MOLECULAR GENETICS, Volume 17 Number 5, issued September 1991, L. Pouncey et al., " $\beta$ 1-4-galactosyltransferase gene expression is regulated during entry into the cell cycle and during the cell cycle", pages 435-443, see entire document.	1-5
Y,P	TRANSPLANTATION PROCEEDINGS, Volume 27, number 1, issued February 1995, K.M. Strahan et al., "Pig $\alpha$ 1, 3galactosyltransferase: sequence of a full-length cDNA clone, chromosomal localisation of the corresponding gene, and inhibition of expression in cultured pig endothelial cells", pages 245-246, see entire document.	1-5

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## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-5

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
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## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-5, drawn to transgenic swine comprising antisense nucleic acids.

Group II, claims 1, and 6-9, drawn to transgenic swine comprising ribozyme encoding nucleic acids.

Group III, claims 1, 10, and 16-18, drawn to swine harboring genes mutated by homologous recombination.

Group IV, claims 11-15, drawn to nucleic acids encoding alpha (1,3) galactosyltransferase.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of groups I-III relates to the use of differing means to modify swine so as to alter the expression of alpha (1,3) galactosyltransferase. In the invention of group I, this special technical feature is the use of antisense transgenes that inhibit protein expression at the transcriptional or translational level. In the invention of group II, the special technical feature is the use of ribozyme encoding nucleic acids that involve the use of catalytic RNA species. In the invention of group III, the special technical feature is the direct alteration of endogenous genes. Each of these three special technical features involves diverse and separate areas of consideration. For example, the use of antisense nucleic acids requires consideration of sequence selection and nucleic acid binding; the use of ribozyme encoding nucleic acids requires consideration of design and expression of catalytic nucleic acids; and the use of animals harboring experimentally induced gene mutations requires consideration of means of performing in vivo site directed mutagenesis. Therefore, although related, the three groups of inventions are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

The special technical feature of the invention of group IV is nucleic acids encoding alpha (1,3) galactosyltransferase. This special technical feature bears no requisite relationship to any transgenic animals such as those defined by the inventions of groups I-III. Further, nucleic acids represent materially different compositions than those of multicellular animals.

For the preceding reasons, the inventions listed above are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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